Part 1

Role of the cytoplasmic microtubules on the behavior of meiotic products during conjugation in *Paramecium caudatum*

Abstract

To make the dynamics of microtubules clear at the stage of the selection of meiotic products in *Paramecium caudatum*, the localization of microtubules was analyzed by indirect immunofluorescence using a monoclonal anti-α-tubulin antibody. After meiosis, four haploid meiotic products are formed in a conjugating cell. The immunofluorescence showed that the cytoplasmic microtubules appeared between some meiotic products and the paroral region, and then were assembled around a meiotic product survived in the region. Furthermore, by injection of the anti-α-tubulin antibody, the nuclear movement to the paroral region was inhibited, and all the meiotic products degenerated. These results suggest that the cytoplasmic microtubules are essential for the migration of meiotic products to the paroral region.

Introduction

In *Paramecium*, as well as in yeast, the nuclear envelope does not break down during nuclear division; the mitotic spindle forms inside the nucleus. In yeast *Saccharomyces cervisiae*, microtubules extensively work in nuclear migration, nuclear division and karyogamy (Delgado and Conde, 1984; Huffaker *et al.*, 1988). In *Paramecium* (Mikami, 1980; Grandchamp and Beisson, 1981), elongation of the spindles results in orienting the daughter nuclei anteroposteriorly, and this orientation determines the nuclear differentiation either to macronuclear anlagen or micronuclei.

Sexual cycle of *Paramecium* has two stages of nuclear movement. One of them is a selection of meiotic products. During conjugation in *P. caudatum*, a micronucleus undergoes meiosis and produces four haploid nuclei. One of the four nuclei lies in the paroral region that locates around the degenerated oral apparatus and survives, while the other three nuclei degenerate (Wichterman, 1946; Sonneborn, 1954; Skoblo and Ossipov, 1968). As regards the nuclear selection after meiosis, Yanagi and Hiwatashi (1985) showed that microtubules are involved in the migration of meiotic products into the paroral region by the inhibition experiment of treatment with vinblastine on *P. caudatum*. However, they did not show how and when microtubules contribute to the nuclear migration.

In the part 1, I focused on microtubule dynamics at the stage of the selection of meiotic products in *P. caudatum*. This part will be show that the cytoplasmic microtubules are essential for the migration of meiotic products during conjugation. Results obtained also suggest that the cytoplasmic microtubules are involved in the survival of meiotic products.

Materials and Methods

Stocks and culture. The stocks used were C103s8 selfing progeny of C103, mating type VI (kindly supplied by Dr. T. Watanabe, Tohoku University), 16BKy-9 and 16BKy-13, progeny of cross between 16B803 and Kyk402, mating type V belonging to syngen 3 of *Paramecium caudatum*. Culture and handling techniques for paramecia followed those of Hiwatashi (1968). The culture medium was fresh lettuce juice diluted with Dryl's solution (Dryl, 1959) modified by the substitution of KH_2PO_4 for NaH_2PO_4 (K-DS) and inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* as food organisms the day before use. Cultivation and experiments were carried out at 25±1℃.

Concentration of conjugating pairs. Conjugation was induced by the method of Hiwatashi (1968). For concentration of conjugating cells, the techniques followed these of Yang and Takahashi (1999). Two h after inducing of conjugation, several drops of iron dextran particles were added to the concentrated cell suspension (Vosskuhlerk and Tiedtke, 1993). Conjugating cells can swim freely under a magnetic force, because they cannot form food vacuoles containing iron dextran particles. After incubation with the iron dextran particles for 5 min, the cells were subjected to a magnetic force to remove unconjugating cells forming food vacuoles. Then the mating pairs were rinsed with fresh K-DS four times by hand centrifugation, and incubated.

Electrophoresis and immunoblotting. SDS-PAGE was carried out according to the method of Laemmli (1970). Isolation of cilia was performed by the modified method of Takahashi *et al.* (1974). Cells and isolated cilia were solubilized with 8 M guanidine HCl containing 10% 2-mercaptoethanol and 0.1 M Tris-HCl (pH 7.5), and dialyzed against 7 M

urea (Hirabayashi *et al.*, 1983). Each protein sample was subjected to 10% SDS-PAGE. Proteins were stained with a Silver stain kit (Wako Chemicals Ltd.). For immunoblotting analysis, proteins resolved by SDS-PAGE were transferred onto PVDF membrane (Immobilion, Millipore), which was incubated with a monoclonal anti-chick α-tubulin antibody (mAb N356, Amersham Pharmacia Biotech, 1:800 dilution). The immunoblots were visualized with alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Biosource, 1:1000 dilution), using a BCIP/NTB phosphatase system (Kirkegaard and Perry Laboratories).

Iimmunofluorescence. Immunofluorescence was performed by the modified method of Ishida *et al.* (1999). Cells were fixed with 1.5% formaldehyde in a PHEM buffer, pH 7.0 (60mM PIPES, 25mM HEPES, 10mM EGTA, 2mM $MgCl_2$) as described by Schliwa and Van Blerkom (1981) at room temperature for 45 min. After fixation, the cells were permeabilized with cold acetone at -20℃ for 20 min. The cells were washed five times with the PBS (pH 7.0) containing 2mM $MgCl_2$ and 10mM EGTA (PBSm) as described by Keryer *et al.* (1989) and then transferred into the PBSm containing 1% BSA. The cells were incubated with the monoclonal anti-chick α-tubulin antibody (1:100 dilution with PBSm containing 0.5% BSA, Amersham Phermacia Biotech) for 1 h at room temperature. After washing four times with PBSm, the cells were incubated with the secondary antibody consisted of FITC-conjugated goat anti-mouse IgG (1:100 dilution, Kappel) for 2 h at room temperature and then washed three times with PBSm. In order to visualize DNA, the cells were stained with 5 μ g/ml PI after treated with RNase (4 mg/ml). Finally, the cells were mounted in VECTA SHIELD Mounting Medium (Vector Laboratories) and observed under a laser scanning confocal microscope (Lsm 410, Zeiss). Each fluorescent section was scanned individually to avoid bleed-through between channels. Each image was

constructed about 10 sections. Each Z axis of sections was 1 μ m.

Microinjection. At the stage of the second meiotic division, conjugating pairs were isolated and one cell of each mating pair was injected with the monoclonal anti- α -tubulin antibody (20 to 30 pl) (Koizumi, 1974). After the injection, cells were placed in K-DS supplemented with BSA (1 mg/ml) for 20 min and then transferred into BSA free K-DS. Micronuclear behavior was observed using a negative contrast phase microscope (Nikon) with a moist chamber (Mikami, 1992). Mating pairs were put onto a cover glass with an amount of K-DS containing 10 mg/ml BSA and were flattened on the cover glass by removing most of the solution surrounding the cells with a micropipet so that they were prevented from swimming. To prevent the cells from drying up, the cover glass was turned over and quickly placed over the moist chamber. The cells were observed every 20 min.

Results

Intracellular localization of α-tubulin after the second meiotic division.

Immunoblotting analysis was used to verify the specificity of a monoclonal antibody raised against the chick α-tubulin. This antibody recognized a single band, corresponding to α-tubulin of *P. caudatum*, on a Western blot containing total or ciliary proteins of *P. caudatum* (Fig. 2).

At the time of the second meiotic division, micronuclei and their separation spindles show immunofluorescence for α -tubulin, though the macronucleus is not decorated (Fig. 3 A). The decoration must be specific to the antibody because no decoration was observed when the mating pairs were reacted with the secondary antibody without the anti-α-tubulin antibody (data not shown). The oral apparatus are degenerating during meiosis (Fig. 3 A, arrows). Then the region around degenerated the oral apparatus develop to the paroral region (Fig. 3 B, between asterisks). All of the micronuclei moved to gather around the paroral region after meiosis (Fig. 3 B, right cell). The cytoplasmic microtubules are observed between the paroral region and some meiotic products at that time (Fig. 3 B, arrow). The prospective surviving meiotic product located in the paroral region is surrounded by the cytoplasmic microtubules (Fig. 3 B, arrowhead, left cell). Then the cytoplasmic microtubules are radially assembled around a survived meiotic product in the paroral region (Fig. 3 C, arrowheads). The other three meiotic products not surrounded by the cytoplasmic microtubules degenerate (Fig. 3 C, dm).

Effect of the antibody on migration of meiotic products.

To probe the role of microtubules on the migration of meiotic products, the anti-α-tubulin antibody was injected into the cytoplasm of conjugating cell at the second meiotic division. The injected cells were observed under a phase-contrast microscope every 20 min for 4 h after the injection.

When BSA (1 mg/ml, estimated to be 20 to 30 pl) as a control was injected into the cytoplasm of the conjugating cells at the second meiotic division, behavior of meiotic products was not influenced (Table 1). That is, all four meiotic products gathered around the paroral region, and then one of them moved into the paroral region and survived there. The clear area was formed around the surviving meiotic product in the paroral region. The clear area seemed to consist with the assembly of the cytoplasmic microtubules around the survived meiotic product. When the pairs were injected with the anti-α-tubulin antibody (protein; 160 μg/ml, estimated to be 20 to 30 pl) at the same stage as control, the migration of the four meiotic products to the paroral region was inhibited, though the second meiotic division was not inhibited. In these pairs, no meiotic product moved into the paroral region (Table 1). All meiotic products gathered in a central area and did not move to the paroral region. About 4 h after the injection, all meiotic products became pycnotic and degenerated. The clear area never appeared around the paroral region in the cell injected with the anti-α-tubulin antibody.

Discussion

Cytoplasmic microtubules are necessary for migration of meiotic products.

In the present study, it was confirmed that when all meiotic products gathered to the paroral region some of the meiotic products were connected to the paroral region by the cytoplasmic microtubules (Fig. 3 B, right cell; Fig. 4 A). The cytoplasmic microtubules were observed to surround the prospective surviving meiotic product (Fig. 3 B, left cell; Fig. 4 B). Moreover, this migration was inhibited by the injection of the anti-α-tubulin antibody (Table 1). In the cells injected with the anti-α-tubulin antibody, all of the meiotic products gathered at a central area in the cell but not in the paroral region, although meiotic division underwent. This result showed that the injection of anti-α-tubulin antibody into the cytoplasm may inhibit the polymerization of cytoplasmic microtubules and not of intranuclear microtubules. Penetration of molecules of the antibody into the nuclei may be prevented by the nuclear envelope. These results suggest that the cytoplasmic microtubules play an important role in migration of meiotic products to the paroral region. However, the nuclear gathering in a central area of the cell was not inhibited by the injection of the anti-α-tubulin antibody. This result may show that some factors other than microtubules are involved in the initial location of meiotic products. In *Tetrahymena*, the network structures composing of the intermediate filament protein (49-kDa protein) spread from the cell-cell junction to the macro- and micronuclei (Numata *et al.*, 1985; Takagi *et al.*, 1991). Whether the similar kinds of protein may be concerned with the movement of meiotic products in *Paramecium*, will be interesting problems.

Cytoplasmic microtubules are involved in the survival of meiotic products.

The migration of meiotic products into the paroral region in *Paramecium* (Wichterman, 1946; Sonneborn, 1954; Skoblo and Ossipov, 1968) and the janctional area in *Tetrahymena* (Nanney, 1953) is essential for their survival at gametogenesis. Gaertig and Fleury (1992) suggested that microtubules seemed to anchor the survived meiotic product to the junctional area in *T. thermophila*. In *P. caudatum*, I first visualized that the surviving micronucleus was surrounded by the cytoplasmic microtubules radially assembled in the paroral region (Figs. 3 C and 4 C). Those results show that the cytoplasmic microtubules are involved in the survival of meiotic products in ciliates.